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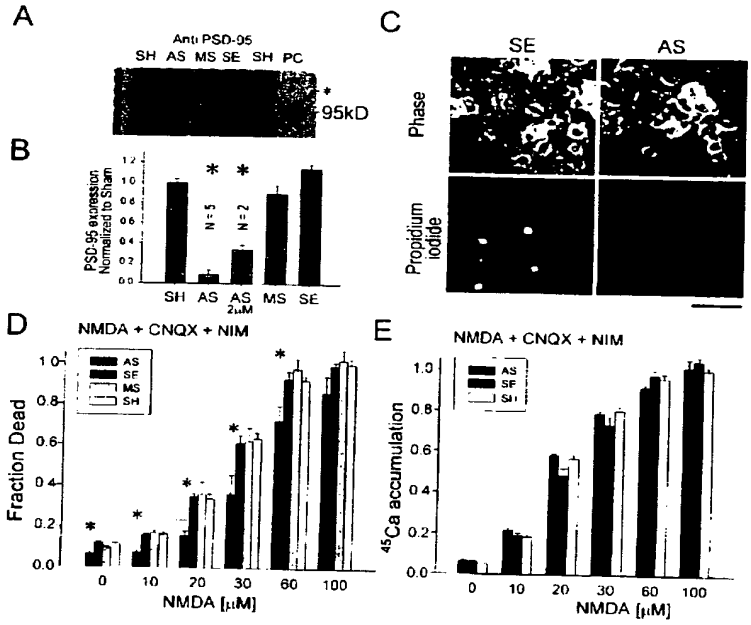
(54) **METHODE POUR REDUIRE LES DOMMAGES AUX CELLULES DE MAMMIFERES**

(54) **METHOD OF REDUCING INJURY TO MAMMALIAN CELLS**




(57) A method of inhibiting the binding between N-methyl-D-aspartate receptors and neuronal proteins in a neuron the method comprising administering to the neuron an effective inhibiting amount of a peptide replacement agent for the NMDA receptor neuronal protein interaction domain or precursor therefor to effect the inhibition. The method is of value in reducing the damaging effect of injury to mammalian cells.

## ABSTRACT

A method of inhibiting the binding between N-methyl-D-aspartate receptors and neuronal proteins in a neuron the method comprising administering to the neuron an effective inhibiting amount of a peptide replacement agent for the NMDA receptor neuronal protein interaction domain or precursor therefor to effect the inhibition. The method is of value in reducing the damaging effect of injury to mammalian cells.

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# METHOD OF REDUCING INJURY TO MAMMALIAN CELLS

## FIELD OF THE INVENTION

This invention relates to methods of reducing the damaging effect of an injury to mammalian cells by treatment with compounds which reduce the binding between N-methyl-D-aspartate receptors and neuronal proteins; pharmaceutical compositions comprising said compounds and methods for the preparation of said pharmaceutical compositions.

## BACKGROUND TO THE INVENTION

Ischemic or traumatic injuries to the brain or spinal cord often produce irreversible damage to central nervous system (CNS) neurons and to their processes. These injuries are major problems to society as they occur frequently, the damage is often severe, and at present there are still no effective treatments for acute CNS injuries. Clinically, ischemic cerebral stroke or spinal cord injuries manifest themselves as acute deteriorations in neurological capacity ranging from small focal defects, to catastrophic global dysfunction, to death. It is currently felt that the final magnitude of the deficit is dictated by the nature and extent of the primary physical insult, and by a time-dependent sequence of evolving secondary phenomena which cause further neuronal death. Thus, there exists a theoretical time-window, of uncertain duration, in which a timely intervention might interrupt the events causing delayed neurotoxicity. However, little is known about the cellular mechanisms triggering and maintaining the processes of ischemic or traumatic neuronal death, making it difficult to devise practical preventative strategies. Consequently, there are currently no clinically useful treatments for cerebral stroke or spinal cord injury.

In vivo, a local reduction in CNS tissue perfusion mediates neuronal death in both hypoxic and traumatic CNS injuries. Local hypoperfusion is usually caused

by a physical disruption of the local vasculature, vessel thrombosis, vasospasm, or luminal occlusion by an embolic mass. Regardless of its etiology, the resulting ischemia is believed to damage susceptible neurons by impacting adversely on a variety of cellular homeostatic mechanisms. Although the nature of the exact disturbances is poorly understood, a feature common to many experimental models of neuronal injury is a rise in free intracellular calcium concentration ($[Ca^{2+}]i$). Neurons possess multiple mechanisms to confine $[Ca^{2+}]_i$ to the low levels, about 100nM necessary for the physiological function. It is widely believed that a prolonged rise in $[Ca^{2+}]_i$ deregulates tightly-controlled $Ca^{2+}$-dependent processes, causing them to yield excessive reaction products, to activate normally quiescent enzymatic pathways, or to inactivate regulatory cytoprotective mechanisms. This, in-turn, results in the creation of experimentally observable measures of cell destruction, such as lipolysis, proteolysis, cytoskeletal breakdown, pH alterations and free radical formation.

The classical approach to preventing $Ca^{2+}$ neurotoxicity has been through pharmacological blockade of $Ca^{2+}$ entry through $Ca^{2+}$ channels and/or of excitatory amino acid (EAA) – gated channels. Variations on this strategy often lessen EAA - induced or anoxic cell death in vitro, lending credence to the $Ca^{2+}$-neurotoxicity hypothesis. However, a variety of $Ca^{2+}$ channel- and EAA- antagonists fail to protect against neuronal injury in vivo, particularly in experimental Spinal Cord Injury (SCI), head injury and global cerebral ischemia. It is unknown whether this is due to insufficient drug concentrations, inappropriate $Ca^{2+}$ influx blockade, or to a contribution from non-$Ca^{2+}$ dependent neurotoxic processes. It is likely that $Ca^{2+}$ neurotoxicity is triggered through different pathways in different CNS neuron types. Hence, successful $Ca^{2+}$-blockade would require a polypharmaceutical approach.

As a result of investigations, I have discovered methods of reducing the damaging effect of an injury to mammalian cells by treatment with compounds to reduce the binding between N-methyl-D-aspartate (NMDA) receptors and neuronal proteins.

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## SUMMARY OF THE INVENTION

It is a preferred object of the present invention to provide in its broadest aspect a method of reducing the damaging effect of an injury to mammalian cells.

In a further preferred object, the invention provides pharmaceutical compositions for use in treating mammals to reduce the damaging effect of an injury to mammalian tissue.

The present invention is based on the discovery of a neuroprotective effect against excitotoxic and ischemic injury by inhibiting the binding between N-methyl-D-aspartate (NMDA) receptors and neuronal proteins in a neuron.

Accordingly, in one aspect the invention provides a method of inhibiting the binding between N-methyl-D-aspartate receptors and neuronal proteins in a neuron said method comprising administering to said neuron an effective inhibiting amount of a peptide replacement agent for the NMDA receptor neuronal protein interaction domineer or precursor therefor to effect said inhibition.

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In a further aspect, the invention provides a method of reducing the damaging effect of ischemia or traumatic injury to the brain or spinal chord in a mammal, said method comprising treating said mammal with a non-toxic, damage-reducing, effective amount of a peptide replacement agent for the NMDA receptor neuronal protein interaction domain or precursor therefor.

The NMDA agent is, preferably, bindable with membrane associated guanylate kinases, and most preferably, is selected from postsynaptic density-95 proteins, PSD-95, PSD-93 and SAP102.

I have found that the replacement agent is a tSXV-containing peptide or precursor therefor, preferably KLSSLESDV.

In a yet further aspect the invention provides a pharmaceutical composition comprising a peptide replacement agent for the NMDA receptor neuronal protein interaction domain or a precursor therefor in a mixture with a pharmaceutically acceptable carrier when used for reducing the damaging effect of an ischemic or traumatic injury to the brain or spinal chord of a mammal; preferably further comprising antessapedia internalisation peptide.

In a further aspect, the invention provides a method of inhibiting the binding between NMDA receptors and neuronal proteins in a neuron, said method comprising administering to said neuron an effective inhibiting amount of an antisense DNA to prevent expression of said neuronal proteins to effect inhibition of said binding. Preferably, this aspect provides a method wherein said antisense DNA reduces the expression of a membrane associated guanylate kinase bindable to said NMDA receptor. More preferably, the guanylate kinase is selected from PSD-95, PSD-93 and SAP102.

In the mammalian nervous system, the efficiency by which *N*-methyl-D-aspartate receptor (NMDAR) activity triggers intracellular signaling pathways governs neuronal plasticity, development, senescence and disease. I have studied excitotoxic NMDAR signaling by suppressing the expression of the NMDAR scaffolding protein PSD-95. In cultured cortical neurons, this selectively attenuated NMDAR excitotoxicity, but not excitotoxicity by other glutamate or $Ca^{2+}$ channels. NMDAR function was unaffected, as receptor expression, while NMDA-currents

and $^{45}Ca$ loading via NMDARs were unchanged. Suppressing PSD-95 selectively blocked $Ca^{2+}$ -activated nitric oxide production by NMDARs, but not by other pathways, without affecting neuronal nitric oxide synthase (nNOS) expression or function. Thus, PSD-95 is required for the efficient coupling of NMDAR activity to nitric oxide toxicity and imparts specificity to excitotoxic $Ca^{2+}$ signaling.

It is known that calcium influx through NMDARs plays key roles in mediating synaptic transmission, neuronal development, and plasticity *(1)*. In excess, Ca influx triggers excitotoxicity *(2)*, a process that damages neurons in neurological disorders that include stroke, epilepsy, and chronic neurodegenerative conditions *(3)*. Rapid $Ca^{2+}$-dependent neurotoxicity is triggered most efficiently when $Ca^{2+}$ influx occurs through NMDARs, and cannot be reproduced by loading neurons with equivalent quantities of $Ca^{2+}$ through non-NMDARs or voltage-sensitive $Ca^{2+}$ channels (VSCCs) *(4)*. This observation suggests that $Ca^{2+}$ influx through NMDAR channels is functionally coupled to neurotoxic signaling pathways.

Without being bound by theory, I believe that lethal $Ca^{2+}$ signaling by NMDARs is determined by the molecules with which they physically interact. The NR2 NMDAR subunits, through their intracellular C-terminal domains, bind to PSD-95/SAP90 *(5)*, chapsyn-110/PSD-93, and other members of the membrane-associated guanylate kinase (MAGUK) family *(6)*. NMDAR-bound MAGUKs are generally distinct from those associated with non-NMDARs *(7)*. I have found that the preferential activation of neurotoxic $Ca^{2+}$ signals by NMDARs is determined by the distinctiveness of NMDAR-bound MAGUKs, or of the intracellular proteins that they bind. PSD-95 is a submembrane scaffolding molecule that binds and clusters NMDARs preferentially and, through additional protein-protein interactions, may link them to intracellular signaling molecules *(8)*. Perturbing PSD-95 would impact on neurotoxic $Ca^{2+}$ signaling through NMDARs.

## BRIEF DESCRIPTION OF THE DRAWINGS

In order that the invention may be better understood preferred embodiments will now be described by way of example only with reference to the accompanying drawings wherein:

Fig.1a is an immunublot;

Fig.1b is a bar chart providing densitometric analysis of PSD-95 expression;

Fig.1c represents representative phase contrast and propidium fluorescence images;

Fig.1d is a bar chart of NMDA concentration against fraction of dead cells;

Fig.1e is a bar chart of NMDA concentration against Calcium accumulation.

Fig.2a1-b2 represent bar charts of selective activations of AMPA/Kainate receptors with Kainate (2a1 and 2-a2); and loadings with Vscc's (2-b1) and calcium loading (2-b2).

Fig.3a-d represent immunoblots (3a); NMSa dose-response curves (3b); NMDA current density measurements (3c); and current/time graph (3d) dialyzed with hucifer yellow; and

Fig.4 bar charts (4a; 4c-4f) and immublot of effect on nNOS expression in cultures; hereinafter better described and explained.

## DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

METHODS:

Cultured cortical neurons were prepared by standard techniques *(4,9)* and switched to serum-free media at 24h [Neurobasal with B27 supplement (Gibco)]. The AS ODN corresponded to nucleotides 435-449 (5'-GAATGGGTCACCTCC-3') of mouse PSD-95/SAP90 mRNA (GeneBank Acc. No. D50621). Filter-sterilized phosphodiester AS (5'-GAATGGGTCACCTCC-3'), SE, and MS (5'-CCGCTCTATCGAGGA-3') ODNs (5μM) were added in culture medium during feedings at 4,6,8 and 10 days after plating. Cultures were used for all experiments

(figs. 1-4) on day 12. ODN sequences exhibited no similarity to any other known mammalian genes (BLAST search *(10)*).

Immunoblotting was done as described in ref. "26". Tissue was harvested and pooled from 2 cultures/lane. The blotted proteins were probed using a monoclonal anti-PSD-95 mouse IgG1 (Transduction Labs, 1:250 dilution), polyclonal anti PSD-93 (1:1000 dilution) and anti SAP-102 (1:2000 dilution) rabbit serum antibodies (Synaptic Systems GmbH), a monoclonal anti NR1 mouse IgG2a (PharMingen Canada, 1:1000 dilution) or a monoclonal anti nNOS (NOS type I) mouse IgG2a (Transduction Labs, 1:2500 dilution). Secondary antibodies were sheep anti-mouse, or donkey anti-rabbit Ig conjugated to horseradish peroxidase (Amersham). Immunoblots for PSD-95 were obtained for all experiments (Figs 1-4) from sister cultures, and all gels quantified using an imaging densitometer (Bio-Rad GS-670).

cGMP determinations were performed 10 min after challenging the cultures with NMDA, kainate, or high-K (Figs. 4c-e) with the Biotrak cGMP enzymeimmunoassay system according to the kit manufacturer's instructions (Amersham). Staining for NADPH diaphorase (Fig 4b) was done as described in ref. 12.

Electrophysiology. Whole cell patch-clamp recordings in the cultured neurons were performed and analyzed as described in ref. 13. During each experiment a voltage step of -10 mV was applied from holding potential and the cell capacitance was calculated by integrating the capacitative transient. The extracellular solution contained (in mM): 140 NaCl, 5.4 KCl, 1.3 $CaCl_2$, 25 HEPES, 33 glucose, 0.01 glycine, and 0.001 tetrodotoxin (pH =7.3-7.4, 320 - 335 mOsm). A multi-barrel perfusion system was employed to rapidly exchange NMDA containing solutions. The pipette solution contained (in mM): 140 CsF, 35 CsOH, 10 HEPES, 11 EGTA, 2 tetraethylammonium chloride (TEA), 1 $CaCl_2$, 4 MgATP, pH 7.3 at 300 mOsm. Lucifer yellow (LY; 0.5% w/v) was included in the pipette for experiments in figure 3d.

Excitotoxicity and $Ca^{2+}$ accumulation measurements were performed identically to the methods described and validated in refs. 4 and 14. We used measurements of propidium iodide fluorescence as an index of cell death, and of radiolabelled $^{45}Ca^{2+}$ accumulation for $Ca^{2+}$ load determinations in sister cultures on the same day. Experimental solutions were as previously described *(4)*. $Ca^{2+}$ influx was pharmacologically channeled through distinct pathways as follows: To NMDARs by applying NMDA (x60 min) in the presence of both CNQX (Research Biochemicals Inc) and nimodipine (Miles Pharmaceuticals), to non-NMDARs by applying kainic acid (x60 min or 24h) in the presence of both MK-801 (RBI) and nimodipine, and to VSCCs using 50 mM $K^+$ solution (x60 min) containing 10mM $Ca^{2+}$ and S(-)-Bay K 8644, an L-type channel agonist (300-500nM; RBI), MK-801 and CNQX. Antagonist concentrations were (in μM): MK-801 10, CNQX 10, nimodipine 2. All three antagonists were added after the 60 min agonist applications for the remainder of all experiments (24 h). A validation of this approach in isolating $Ca^{2+}$ influx to the desired pathway in our cortical cultures has been published *(4)*.

Whole cell patch-clamp recordings in the cultured neurons were performed and analyzed as described in Z. Xiong, W. Lu, J.F. MacDonald, *Proc Natl Acad Sci USA* 94, 7012 (1997). During each experiment a voltage step of -10 mV was applied from holding potential and the cell capacitance was calculated by integrating the capacitative transient. The extracellular solution contained (in mM): 140 NaCl, 5.4 KCl, 1.3 $CaCl_2$, 25 HEPES, 33 glucose, 0.01 glycine, and 0.001 tetrodotoxin (pH =7.3-7.4, 320 - 335 mOsm). A multi-barrel perfusion system was employed to rapidly exchange NMDA containing solutions. The pipette solution contained (in mM): 140 CsF, 35 CsOH, 10 HEPES, 11 EGTA, 2 tetraethylammonium chloride (TEA), 1 $CaCl_2$, 4 MgATP, pH 7.3 at 300 mOsm. Lucifer yellow (LY; 0.5% w/v) was included in the pipette for experiments in figure 3D.

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Data analysis: data in all figures were analyzed by ANOVA, with a post-hoc Student's t-test using the Bonferroni correction for multiple comparisons. All means are presented with their standard errors.

In greater detail:

Figure 1, shows increased resilience of PSD-95 deficient neurons to NMDA toxicity in spite of $Ca^{2+}$ loading. A. Immunoblot showing representative effects of sham (SH) washes, and PSD-95 AS, SE and MS ODNs, on PSD-95 expression. PC: positive control tissue from purified rat brain cell membranes. Asterisk: non-specific band produced by the secondary antibody, useful to control for protein loading and blot exposure times. B. Densitometric analysis of PSD-95 expression pooled from N experiments. Asterisk: different from other groups, one-way ANOVA, F = 102, $p<0.0001$. ODNs were used at 5μM except where indicated (AS 2μM). C. Representative phase contrast and propidium iodide fluorescence images of PSD-95 deficient (AS) and control (SE) cultures 24 h after a 60 min challenge with 30μM NMDA. Scale bar: 100 μm. D. Decreased NMDA toxicity at 24h in PSD-95 deficient neurons following selective NMDAR activation x 60 min (n=16 cultures/bar pooled from N=4 separate experiments). Asterisk: differences from SE, MS and SH (Bonferroni t-test, $p<0.005$). Death is expressed as the fraction of dead cells produced by 100μM NMDA in sham-ODN-treated controls (validated in *4,14*). E. No effect of PSD-95 deficiency on NMDAR-mediated $Ca^{2+}$ loading (n = 12/bar, N = 3; reported as the fraction of $^{45}Ca^{2+}$ accumulation achievable over 60 min in the sham controls by 100μM NMDA, which maximally loads the cells with calcium *(4)*.

Figure 2, shows thatPSD-95 deficiency does not affect toxicity and $Ca^{2+}$ loading produced by activating non-NMDARs and $Ca^{2+}$ channels. Cultures were treated with SH washes or AS or SE ODNs as in Fig. 1. A. Selective activation of AMPA/kainate receptors with kainate in MK-801 (10μM) and nimodipine (NIM; 2μM) produces toxicity over 24h (A1) irrespective of PSD-95 deficiency, with minimal $^{45}Ca^{2+}$ loading (A2). B. Selective activation of VSCCs produces little

toxicity (B1), but significant $^{45}Ca^{2+}$ loading (B2) that is also insensitive to PSD-95 deficiency. n = 4 cultures/bar in all experiments.

Figure 3, shows that there is no effect of perturbing PSD-95 on receptor function. A. Immunoblots of PSD-95 ODN-treated cultures probed for PSD-95, NR1, PSD-93, and SAP-102 using specific antibodies. PC: positive control tissue from purified rat brain cell membranes. B. NMDA dose-response curves and representative NMDA currents (inset) obtained with 3-300μM NMDA. C. NMDA current density measurements elicited with 300μM NMDA (AS: n = 18; SE: n =19; SH: n = 17; one-way ANOVA F=1.10, p=0.34), and analysis of NMDA current desensitization. $I_{ss}$ = steady-state current; $I_{peak}$ = peak current. AS: n=15; SE: n = 16; SH: n = 16 (ANOVA,, F=0.14, p=0.87). Time constants for current decay were AS: 1310 ± 158 ms; SE, 1530 ± 185 ms; SH: 1190 ± 124 ms (ANOVA, F= 1.22, p= 0.31). D. Currents elicited with 300μM NMDA in neurons dialyzed with LY (insert) and 1mM tSXV or control peptide.

Figure 4, shows the effect of coupling of NMDAR activation to nitric oxide signaling by PSD-95. A. L-NAME protects against NMDA toxicity (n = 4, N = 2). Asterisk: difference from 0 μM L-NAME (Bonferroni t-test, $p<0.05$). B. No effect of SH and of PSD-95 AS and MS ODNs on nNOS expression in cultures (immunoblot) and on NADPH diaphorase staining in PSD-95 AS and SE-treated neurons. PC: positive control tissue from purified rat brain cell membranes. C. Effect of isolated NMDAR activation on cGMP formation (n=12 cultures/bar pooled from N=3 separate experiments) D,E. Effects of VSCC activation (n = 8/bar, N = 2), and AMPA/kainate receptor activation (n = 4/bar, N=1) on cGMP formation. Data in C-E are expressed as the fraction of cGMP produced in SE-treated cultures by 100 μM NMDA. Asterisk: differences from both SH and SE controls (Bonferroni t-test, $p<0.0001$). F. Sodium nitroprusside toxicity is similar in PSD-95 AS, SE and SH treated cultures.

PSD-95 expression was suppressed in cultured cortical neurons to < 10% of control levels, using a 15-mer phosphodiester antisense (AS) oligodeoxynucleotide (ODN) (Fig. 1A,B) Sham (SH) washes, sense (SE) and missense (MS) ODNs *(9)*

had no effect. The ODNs had no effect on neuronal survivability and morphology as gauged by viability assays, herein below, and phase-contrast microscopy (not shown).

To examine the impact of PSD-95 on NMDAR-triggered excitotoxicity, ODN-treated cultures were exposed to NMDA (10-100 μM) for 60 min, washed, and either used for $^{45}Ca^{2+}$ accumulation measurements, or observed for a further 23 h. $Ca^{2+}$ influx was isolated to NMDARs by adding antagonists of non-NMDARs and $Ca^{2+}$ channels *(4)*. NMDA toxicity was significantly reduced in neurons deficient in PSD-95 across a range of insult severities (Figs. 1C,D; $EC_{50}$: AS: 43.2 ± 4.3; SE: 26.3 ± 3.4, Bonferroni t-test, $p < 0.005$). Concomitantly however, PSD-95 deficiency had no effect on $Ca^{2+}$ loading into identically treated sister cultures (Fig. 1E). Therefore, PSD-95 deficiency induces resilience to NMDA toxicity despite maintained $Ca^{2+}$ loading.

I next examined whether the increased resilience to $Ca^{2+}$ loading in PSD-95 deficient neurons was specific to NMDARs. Non-NMDAR toxicity was produced using kainic acid (30-300 μM), a non-desensitizing AMPA/kainate receptor agonist *(15)*, in the presence of NMDAR and $Ca^{2+}$ channel antagonists *(4)*. Kainate toxicity was unaffected in PSD-95 deficient in neurons challenged for either 60 min (not shown) or 24 h (Fig. 2A1). Non-NMDAR toxicity occurred without significant $^{45}Ca^{2+}$ loading (Fig. 2A2), as >92% of neurons in these cultures express $Ca^{2+}$-impermeable AMPA receptors *(4)*. However, $Ca^{2+}$ loading through VSCCs, which is non-toxic *(4)* (Fig. 2B1), was also unaffected by PSD-95 deficiency (Fig. 2B2). Thus, suppressing PSD-95 expression affects neither toxicity nor $Ca^{2+}$ fluxes triggered through pathways other than NMDARs.

Immunoblot analysis *(11)* of PSD-95 deficient cultures revealed no alterations in the expression of the essential NMDAR subunit NR1, nor of two other NMDAR-associated MAGUKs, PSD-93 and SAP-102 (Fig. 3A). This indicated that altered expression of NMDARs and their associated proteins was unlikely to explain

reduced NMDA toxicity in PSD-95 deficiency (Fig. 1C,D). Therefore, I examined the possibility that PSD-95 modulates NMDAR function. NMDA currents were recorded using the whole-cell patch technique *(16)* (Fig. 3B). PSD-95 deficiency had no effect on passive membrane properties, including input resistance and membrane capacitance [Capacitance: AS 55.0 ± 2.6 pF (n =18 ); SE 52.7 ± 3.2 pF (n=19); SH 48.1 ± 3.4 pF (n = 17; ANOVA, F=1.29, p=0.28)]. Whole-cell currents elicited with 3-300 μM NMDA were also unaffected. Peak currents were AS: 2340 ± 255 pA (n=18); SE: 2630 ± 276 (n=19); SH: 2370 ± 223 (n=17) (Fig. 3B, inset; one-way ANOVA, F = 0.43, p = 0.65). NMDA dose-response relationships also remained unchanged (Fig. 3B; $EC_{50}$ AS: 16.1 ± 0.8 μM (n=7); SE: 15.5 ± 2.1 (n=6); SH: 15.9 ± 2.9; one-way ANOVA, F= 0.02, p = 0.98), as were NMDA current density and desensitization (Figs. 3C).

To further examine the effect of PSD-95 binding on NMDAR function, a 9 aa peptide (KLSSIESDV) corresponding to the C-terminal domain of the NR2B subunit characterized by the tSXV motif *(6)* was injected into the neurons. At 0.5mM, this peptide competitively inhibited the binding of PSD-95 to GST-NR2B fusion proteins *(6)*, and was therefore predicted to uncouple NMDARs from PSD-95. Intracellular dialysis of 1mM tSXV or control peptide (CSKDTMEKSESL) *(6)* was achieved through patch pipettes (3-5 MΩ) also containing the fluorescent tracer Lucifer Yellow (LY). This had no effect on NMDA currents over 30 min despite extensive dialysis of LY into the cell soma
and dendrites (Fig. 3D). Peak current amplitudes were tSXV: 2660 ± 257 pA (n= 9), control: 2540 ± 281 pA (n= 10; $t_{(17)} = 0.31$, p = 0.76).

The data is consistent with that obtained from recently generated mutant mice expressing a truncated 40K PSD-95 protein that exhibited enhanced LTP and impaired learning *(17)*. Hippocampal CA1 neurons in PSD-95 mutants exhibited no changes in NMDAR subunit expression and stoichiometry, cell density, dendritic cytoarchitecture, synaptic morphology, or NMDAR localization using NR1

immunogold labeling of asymmetric synapses. NMDA currents, including synaptic currents, were also unchanged *(16)*. I also found no effects of PSD-95 deficiency on NMDAR expression, on other NMDAR associated MAGUKs, nor on NMDA-evoked currents. In addition, NMDAR function gauged by measuring NMDA-evoked $^{45}Ca^{2+}$-accumulation was unaffected. Thus, the neuroprotective consequences of PSD-95 deficiency must be due to events downstream from NMDAR activation, rather than to altered NMDAR function.

The second PDZ domain of PSD-95 binds to the C-terminus of NR2 subunits and to other intracellular proteins *(8)*. Among these is nNOS *(18)*, an enzyme that catalyzes the production of nitric oxide (NO), a short-lived signaling molecule that also mediates $Ca^{2+}$-dependent NMDA toxicity in cortical neurons *(12)*. Although never demonstrated experimentally, the NMDAR/PSD-95/nNOS complex was postulated to account for the preferential production of NO by NMDARs over other pathways *(8)*. To determine whether NO signaling plays a role in NMDA toxicity in the present cultures, we treated the cells with $N^G$-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor *(12)*. L-NAME protected the neurons against NMDA toxicity (Fig. 4A), indicating the possibility that suppressing PSD-95 might perturb this toxic signaling pathway.

The effect of suppressing PSD-95 expression on NO signaling and toxicity was examined using cGMP formation as a surrogate measure of NO production by $Ca^{2+}$-activated nNOS *(20,21)*. PSD-95 deficiency had no impact on nNOS expression (Fig. 4B), nor on the morphology (Fig. 4B) or counts of NADPH diaphorase-staining *(12)* neurons (SH: 361 ± 60, SE: 354 ± 54, AS: 332 ± 42 staining neurons /10mm coverslip, 3 coverslips/group). However, in neurons lacking PSD-95 challenged with NMDA under conditions that isolated $Ca^{2+}$ influx to NMDARs *(4)*, cGMP production was markedly attenuated (>60%; Fig. 4C, one-way ANOVA, $p<0.0001$). Like inhibited toxicity (Figs. 1,2), inhibited cGMP formation in neurons lacking PSD-95 was only observed in response to NMDA. It was unaffected in neurons

loaded with $Ca^{2+}$ through VSCCs (Fig. 4D), even under high neuronal $Ca^{2+}$ loads matching those attained by activating NMDARs (compare Figs. 1E and 2B2) *(4)*. nNOS function therefore, was unaffected by PSD-95 deficiency. AMPA/kainate receptor activation failed to load the cells with $Ca^{2+}$ (Fig. 2A2), and thus failed to increase cGMP levels (Fig. 4E). Our findings indicate that suppressing PSD-95 selectively reduces NO production efficiency by NMDAR-mediated $Ca^{2+}$ influx, but preserves NO production by $Ca^{2+}$ influx through other pathways.

Bypassing nNOS activation with NO donors restored toxicity in neurons lacking PSD-95. The NO donors sodium nitroprosside *(12)* (Fig. 4F; $EC_{50}$ 300 μM) and *S*-nitrosocysteine *(17)* (not shown) were highly toxic, irrespective of PSD-95 deficiency. Thus, reduced NMDA toxicity in PSD-95 deficient cells was unlikely to be caused by altered signaling events downstream from NO formation.

Suppressing PSD-95 expression uncoupled NO formation from NMDAR activation (Fig. 4C), and protected neurons against NMDAR toxicity (Fig. 1C,D) without affecting receptor function (Figs 1E, 3A-D), by mechanisms downstream from NMDAR activation, and upstream from NO-mediated toxic events (Fig. 4F). Therefore, PSD-95 imparts NMDARs with signaling and neurotoxic specificity through the coupling of receptor activity to critical second messenger pathways. Our results have broader consequences, as NMDAR activation and NO signaling are also critical to neuronal plasticity, learning, memory, and behavior *(1,18,19)*. Thus, our report provides experimental evidence for a mechanism by which PSD-95 protein may govern important physiological and pathological aspects of neuronal functioning.

Although this disclosure has described and illustrated certain preferred embodiments of the invention, it is to be understood that the invention is not restricted to those particular embodiments. Rather, the invention includes all embodiments which are functional or mechanical equivalence of the specific embodiments and features that have been described and illustrated.

SEQUENCE LISTING

1. GENERAL INFORMATION

(i) APPLICANT:
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(ii) TITLE OF INVENTION: METHOD OF REDUCING INJURY TO MAMMALIAN CELLS

(iii) NUMBER OF SEQUENCES: 2

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(v) COMPUTER-READABLE FORM
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC Compatible
(C) OPERATING SYSTEM: MS-DOS Ver. 6.2
(D) SOFTWARE: ASCII Text

(vi) CURRENT APPLICATION DATA
(A) APPLICATION NO.: 2273622
(B) FILING DATE: June 2, 1999

(vii) PATENT AGENT INFORMATION
(A) NAME: SMITH LYONS
(B) REFERENCE NUMBER: CBB1012

2. INFORMATION FOR SEQ ID NO.: 1
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: PRT
(C) ORGANISM: Unknown
(ii) FEATURE:
(A) OTHER INFORMATION: Description of Unknown Organism: peptide

(iii) SEQUENCE DESCRIPTION: SEQ ID NO: 1

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2. INFORMATION FOR SEQ ID NO.: 2
(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: PRT
(C) ORGANISM: Unknown

(ii) FEATURE:
(A) OTHER INFORMATION: Description of Unknown Organism: peptide

(iii) SEQUENCE DESCRIPTION: SEQ ID NO: 2

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I claim:

1. A method of inhibiting the binding between N-methyl-D-aspartate receptors and neuronal proteins in a neuron said method comprising administering to said neuron an effective inhibiting amount of a peptide replacement agent for the NMDA receptor neuronal protein interaction domain or precursor therefor to effect said inhibition.
2. A method as defined in claim 1 wherein said neuron is damaged.
3. A method of reducing the damaging effect of ischemia or traumatic injury to the brain or spinal chord in a mammal, said method comprising treating said mammal with a non-toxic, damage-reducing, effective amount of a peptide replacement agent for the NMDA receptor neuronal protein interaction domain or precursor therefor.
4. A method as defined in claim 3 wherein said mammal is under the influence of neuronal cell damage.
5. A method as defined in any one of claims 1 to 4 wherein said NMDA receptor is bindable with membrane associated guanylate kinases.
6. A method as defined in claim 5 wherein said guanylate kinase is PSD-95.
7. A method as defined in claim 5 wherein said guanylate kinase is PSD-93.
8. A method as defined in claim 5 wherein said guanylate kinase is SAP102.
9. A method as defined in claim 5 wherein said guanylate kinase is SAP97.
10. A method as defined in any one of claims 1 – 9 wherein said replacement agent is a tSXV-containing peptide or precursor therefor.
11. A method as defined in claim 10 wherein said agent is KLSSLESDV or a precursor therefor.
12. A pharmaceutical composition comprising a peptide replacement agent for the NMDA receptor neuronal protein interaction domain in a mixture with a pharmaceutically acceptable carrier or a precursor therefor when used for reducing the damaging effect of an ischemic or traumatic injury to the brain or spinal chord of a mammal.

13. A composition as defined in claim 12 wherein said replacement agent is a tSXV-containing peptide or a precursor therefor.

14. A composition as defined in claim 13 wherein said agent is KLSSLESDV or a precursor therefor.

15. A composition as defined in any one of claims 12 – 14 further comprising antessapedia internalisation peptide.

16. A method of controlling the concentration of $Ca^{2+}$ ions in the vicinity of ion channel pores of cells in vivo to prevent the diffusion of toxic amounts of said $Ca^{2+}$ influx to prevent the triggering of neurotoxic phenomena, said method comprising administering an effective, non-toxic amount of a peptide replacement agent for the NMDA receptor neuronal protein interaction domain or precursor therefor.

17. A method as defined in claim 16 wherein said agent is a tSXV-containing peptide or precursor therefor.

18. A method as defined in claim 17 wherein said agent is KLSSLESDV or a precursor therefor.

19. A process for the manufacture of a pharmaceutical composition as defined in any one of the claims 12 – 15, said process comprising admitting said replacement agent with a pharmaceutically acceptable carrier therefor.

20. A method of inhibiting the binding between NMDA receptors and neuronal proteins in a neuron, said method comprising administering to said neuron an effective inhibiting amount of an antisense DNA to prevent expression of said neuronal proteins to effect inhibition of said binding.

21. A method of reducing the damaging effect of ischemia or traumatic injury to the brain or spinal chord in a mammal, said method comprising treating said mammal with a non-toxic, damage-reducing, effective amount of an antisense DNA to reduce the expression of NMDA receptor binding neuronal proteins and inhibition of binding between NMDA receptors and said neuronal proteins.

22. A method as defined in claim 21 wherein said neuron is damaged.

23. A method as defined in claim 21 and 22 wherein said antisense DNA reduces the expression of a membrane associated guanylate kinases bindable to said NMDA receptor.

24. A method as defined in claim 23 wherein said guanylate kinase is selected from PSD-95 and PSD-93.

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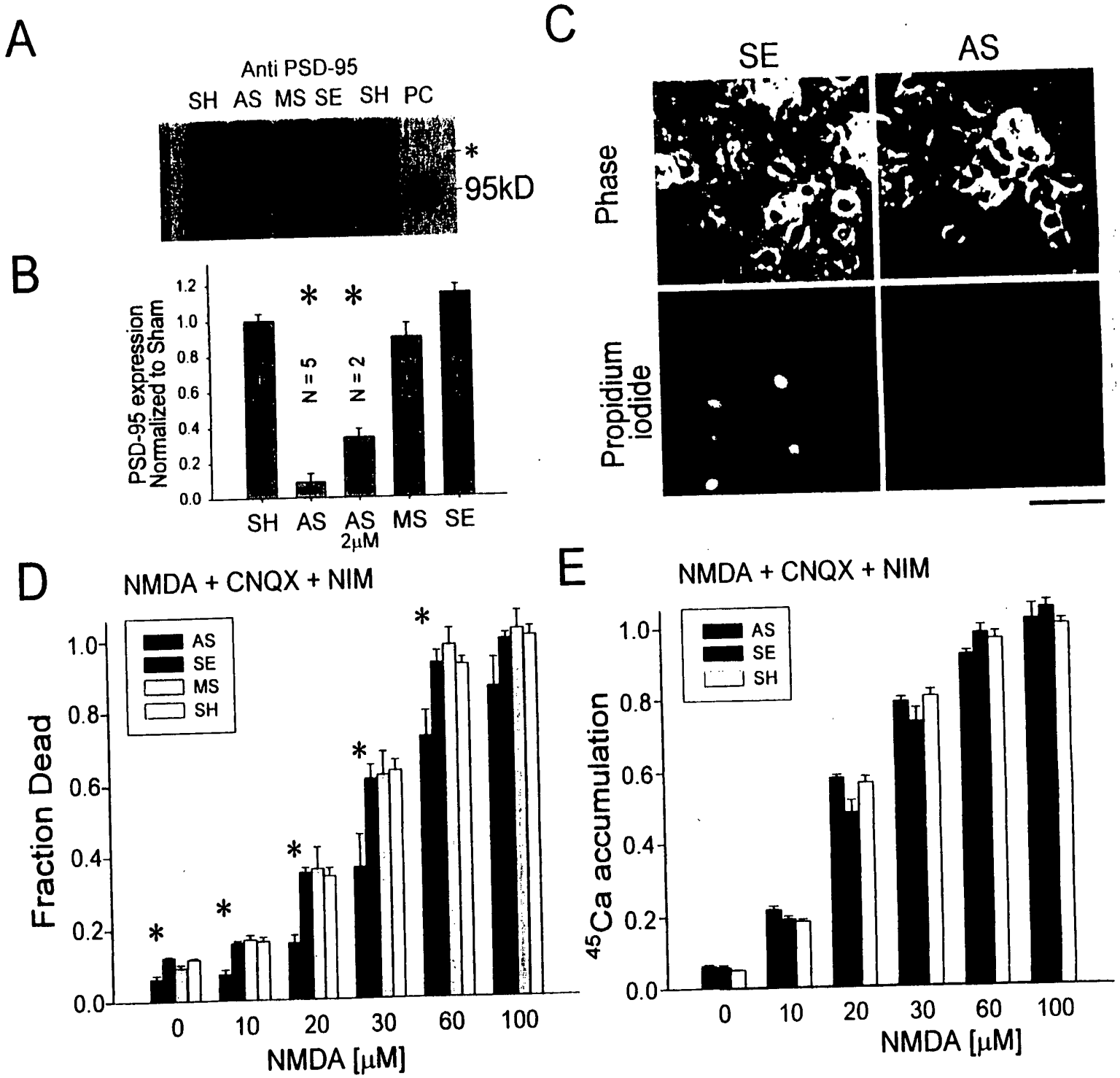


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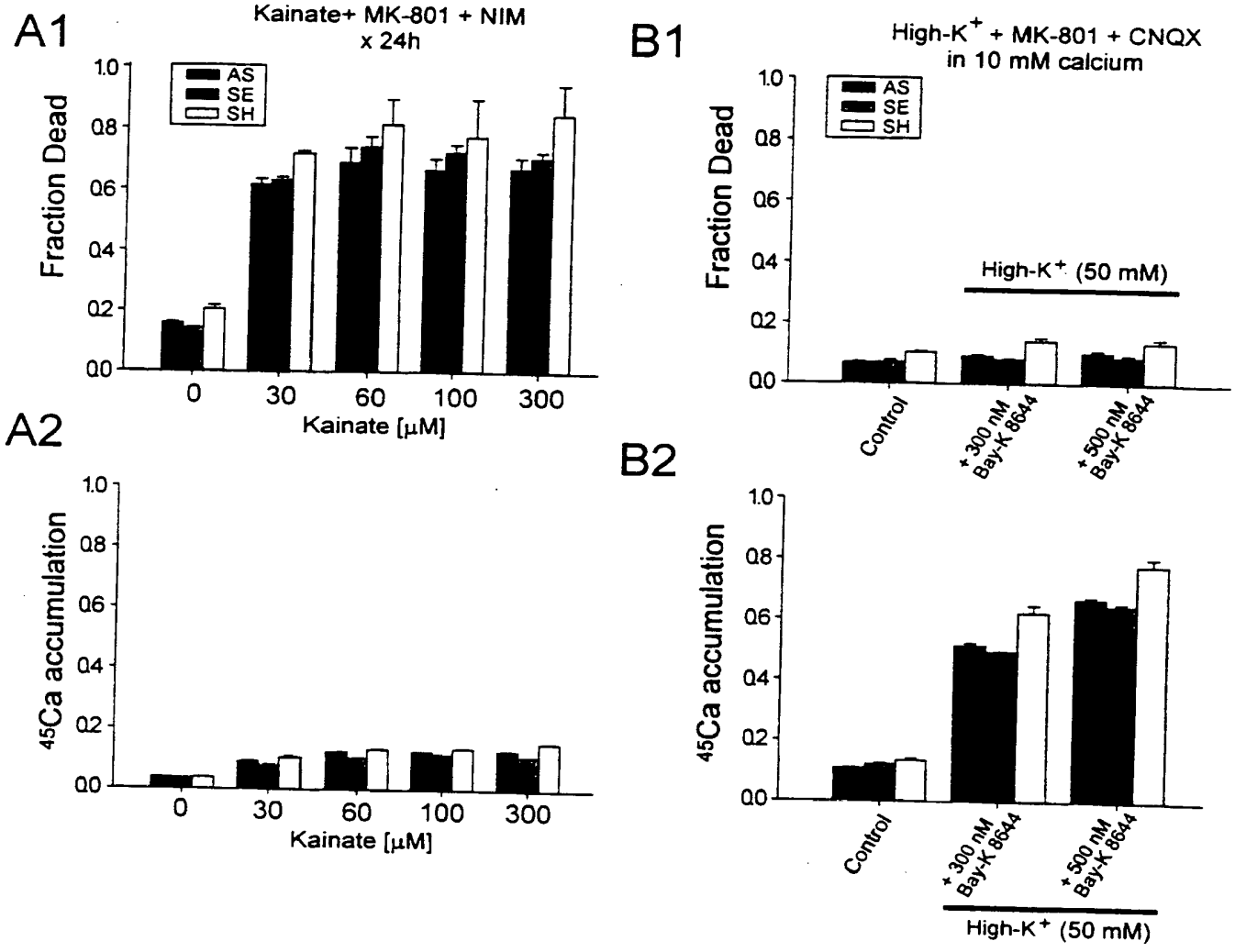
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SE
SH
Fraction Dead
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0.8
0.6
0.4
0.2
0.0
0
30
60
100
300
Kainate [μM]
A2
45Ca accumulation
1.0
0.8
0.6
0.4
0.2
0.0
0
30
60
100
300
Kainate [μM]
B1
High-K+ + MK-801 + CNQX
in 10 mM calcium
AS
SE
SH
Fraction Dead
1.0
0.8
0.6
0.4
0.2
0.0
High-K+ (50 mM)
Control
+ 300 nM Bay-K 8644
+ 500 nM Bay-K 8644
B2
45Ca accumulation
1.0
0.8
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FIG. 2

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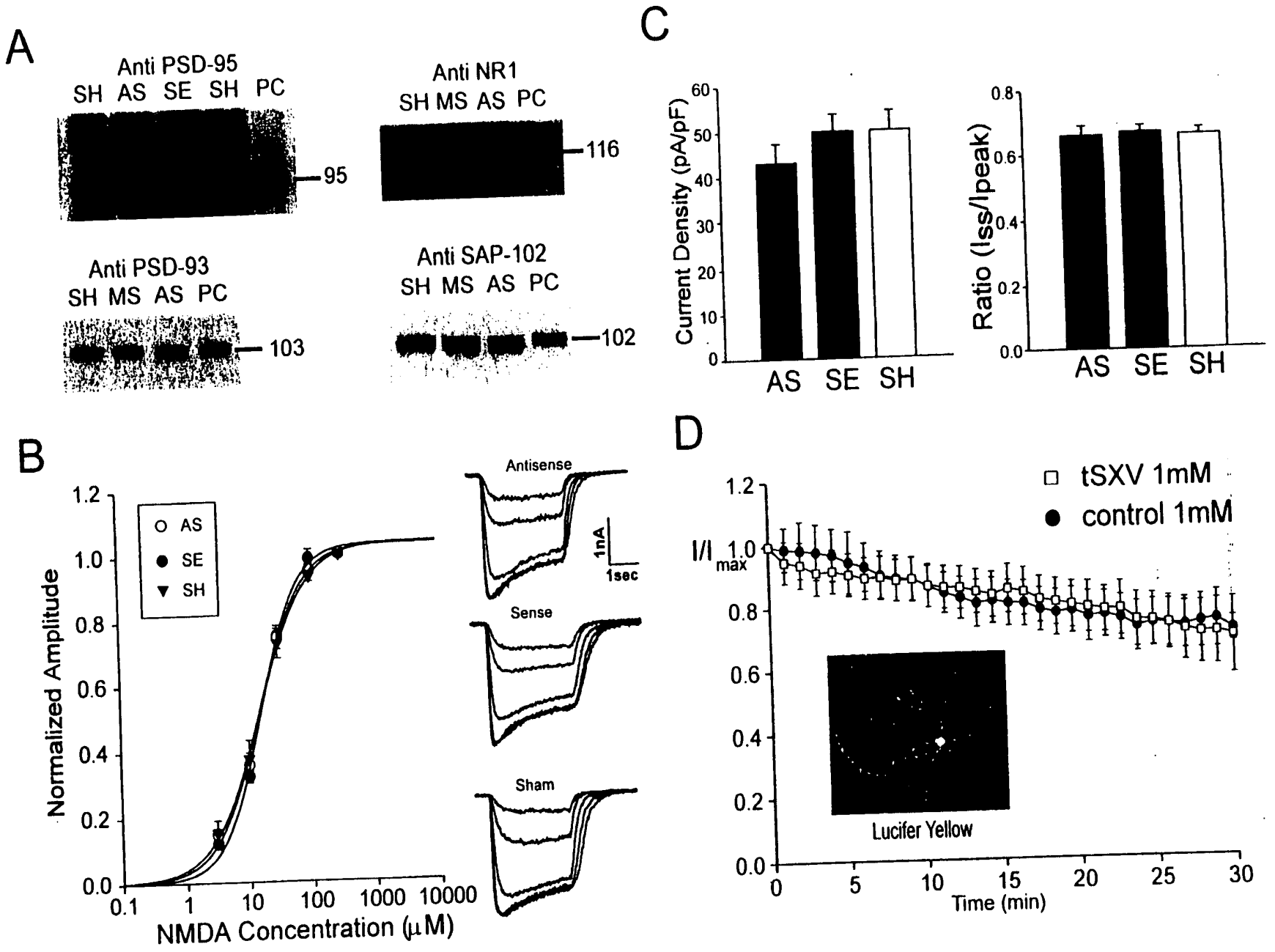


FIG. 3

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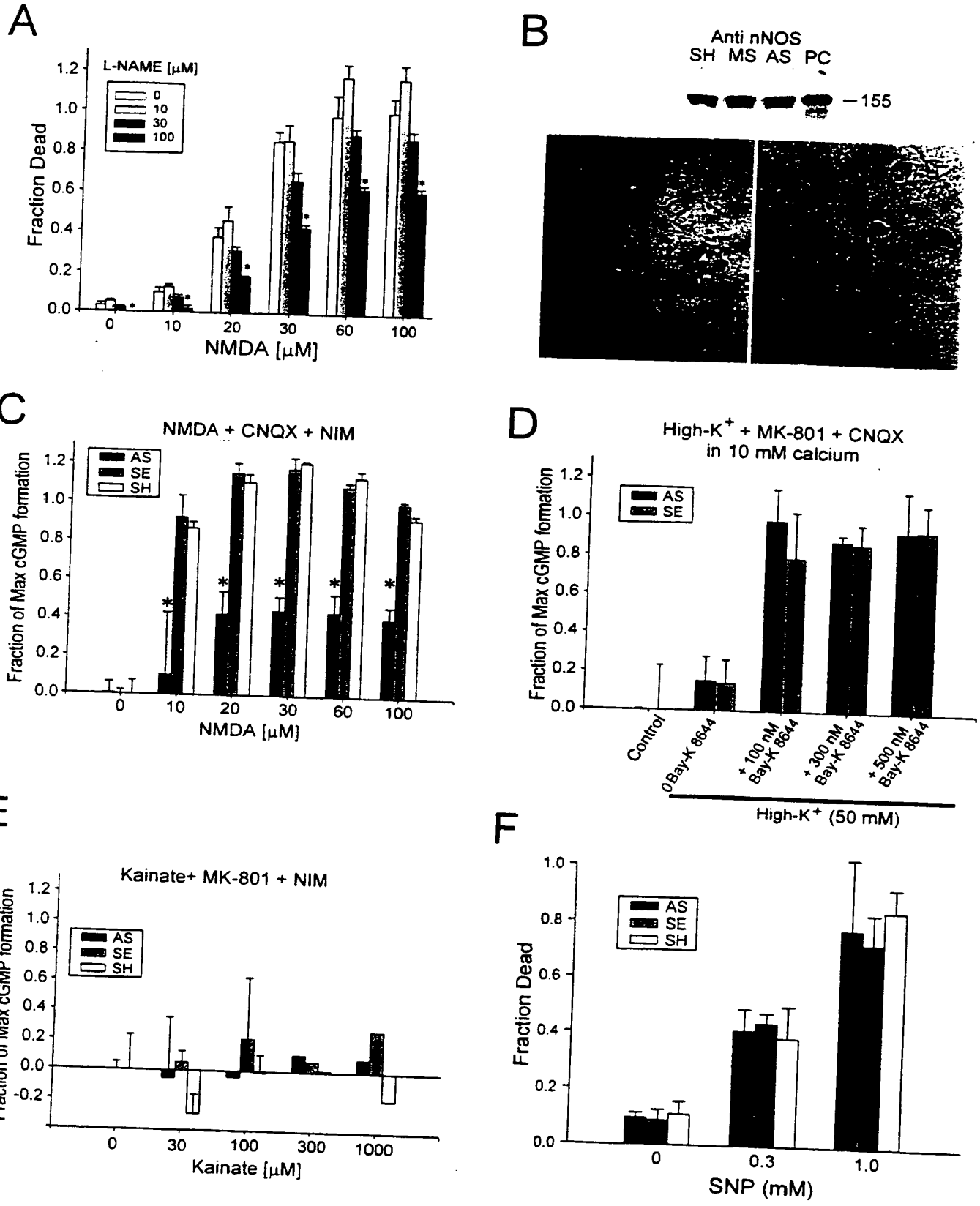
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AS
SE
SH
Fraction of Max cGMP formation
NMDA [μM]
D
High-K+ + MK-801 + CNQX
in 10 mM calcium
AS
SE
Fraction of Max cGMP formation
Control
0 Bay-K 8644
+ 100 nM Bay-K 8644
+ 300 nM Bay-K 8644
+ 500 nM Bay-K 8644
High-K+ (50 mM)
E
Kainate+ MK-801 + NIM
AS
SE
SH
Fraction of Max cGMP formation
Kainate [μM]
F
AS
SE
SH
Fraction Dead
SNP (mM)

FIG. 4